Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 5266–5272, June 1995

Review

Gene targeting approaches to complex genetic diseases: Atherosclerosis and essential hypertension

(animal models/gene disruption/gene duplication/quantitative traits)

Oliver Smithies and Nobuyo Maeda

Department of Pathology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7525

ABSTRACT **Gene targeting allows precise, predetermined changes to be made in a chosen gene in the mouse genome. To date, targeting has been used most often for generation of animals completely lacking the product of a gene of interest. The resulting "knockout" mice have confirmed some hypotheses, have upset others, but have rarely been uninformative. Models of several human genetic diseases have been produced by targeting—including Gaucher disease, cystic fibrosis, and the fragile X syndrome. These diseases are primarily determined by defects in single genes, and their modes of inheritance are well understood. When the disease under study has a complex etiology with multiple genetic and environmental components, the generation of animal models becomes more difficult but no less valuable. The problems associated with dissecting out the individual genetic factors also increase substantially and the distinction between causation and correlation is often difficult. To prove causation in a complex system requires rigorous adherence to the principle that the experiments must allow detection of the effects of changing only a single variable at one time. Gene targeting experiments, when properly designed, can test the effects of a precise genetic change completely free from the effects of differences in any other genes (linked or unlinked to the test gene). They therefore allow proofs of causation.**

Atherosclerosis and essential hypertension are two common diseases that account for a major portion of morbidity and death in the United States. Although population studies have long suggested genetic components in these complex diseases, the details of their inheritance are not well understood, in part because multiple factors are involved, both genetic and environmental. A reasonable working hypothesis is that these diseases in many patients are the consequence of various combinations of genetic defects that individually may have only modest effects. Gene targeting in mice provides a means of testing this hypothesis. Individual genes can be modified and their effects on the development of atherosclerosis or on blood pressures can be evaluated in a manner that excludes the effects of any other genes (linked or unlinked to the target gene). The identification of compensatory mechanisms induced by the primary gene modification can point to other genes that antagonize the effects of the target gene. Combinations of genetic changes can then be made that allow the detection of synergistic or antagonistic interactions. In addition, at any stage of the genetic investigations, the effects of environmental factors such as high cholesterol and fat or salt in the diet can be evaluated, so that the interactions between environment and genotype can be assessed. In what follows, we describe our experiences while using gene targeting to study the genetics of atherosclerosis and hypertension, and some of the challenges encountered, in order to illustrate how such experiments can generate useful animal models for further study and develop an increased understanding of complex genetic diseases.

Atherosclerosis

Atherosclerosis in humans is characterized by the development of intraarterial lesions that may eventually become occlusive (1). The earliest recognizable stage is the preatherosclerotic "fatty streak" in which an aggregation of lipid-rich macrophages can be found in the intima, beneath the endothelial lining of the vessels. Fatty streaks progress to intermediate lesions that have smooth muscle proliferation as well as fatty components. Mature lesions typically have fibrous caps and an acellular lipid core and may develop regions of calcification. Plaque fissuring followed by thrombosis may eventually occur. Of numerous risk factors that have been associated with atherosclerosis, plasma lipoprotein levels are the best studied. Elevated levels of low density lipoprotein (LDL) and reduced levels of high density lipoprotein (HDL) in plasma are correlated with a higher risk for atherosclerosis in humans (2).

No mouse strains are known in which atherosclerosis occurs spontaneously when they are fed regular mouse chow (4.5% fat/0.02% cholesterol). However, experiments by Paigen and her collaborators (3) have shown that some inbred strains develop vascular lesions when fed a high fat (15.8%)/high cholesterol (1.25%) diet. But the lesions, even in a susceptible strain such as C57BL/6J (B6), rarely progress beyond the fatty streak stage. Mice naturally have high plasma levels of HDL and low levels of LDL, conditions that in humans are protective against atherosclerosis. Mice also lack lipoprotein(a), an atherogenic lipoprotein, and their levels of cholesteryl ester transfer protein are extremely low; both these proteins are present in humans. Despite these differences, the general pathways of lipid transport and metabolism in mice and humans are otherwise so similar that one could reasonably expect derangements in specific parts of the pathways to have similar effects in the two species. Nevertheless, when we began our gene targeting experiments in mice, we expected that fully developed atherosclerosis might not be achievable without combining several defects.

A series of targets were initially chosen. The first gene (*Apoe*) codes for apolipoprotein E (apoE), which plays a central role in hepatic clearance from the circulation of chylomicron remnants and very low density lipoprotein remnants. The second gene (*Apoa1*) codes for apolipoprotein A-I (apoA-I), which is the major protein component of HDL. Disruption of the *Apoe* gene was expected to abolish apoE-mediated clearance and therefore lead to an increase in the transit times of lipid in the circulation and to hypercholesterolemia. Removal of apoA-I was expected to cause a reduction in plasma HDL. Because of our uncertainty that atherosclerosis would occur consequent to these primary changes in mice, targeted changes in several other genes were also initiated, including those coding for apoB, apoCIII, and hepatic lipase.

Abbreviations: ACE, angiotensin-converting enzyme; AGT, angiotensinogen; apo, apolipoprotein; HDL, high density lipoprotein; LDL, low density lipoprotein; ANP, atrial natriuretic peptide; ES cell, embryonic stem cell.

ApoE-Deficient Mice Develop Atherosclerosis Spontaneously. Disruption of the *Apoe* gene by us (4) and by Plump and his collaborators at the Rockefeller University (5), in practice, proved by itself to be sufficient to generate a mouse strain in which mature atherosclerotic lesions develop spontaneously. Homozygotes for the disrupted *Apoe* gene (−/−) have severe hypercholesterolemia (4–5 times normal levels); they develop atherosclerosis on normal mouse chow, which is low in fat and cholesterol; and their lesions progress to a complex form (Fig. 1) that is essentially identical to that seen in human patients (6, 7).

The regularity with which advanced atherosclerosis develops spontaneously in the apoE-deficient mice raises at least two questions. First, is apoE deficiency as such important in the etiology of the human disease? Second, is the mouse model of the disease made by this single mutation relevant to the disease in humans, which has a complex genetic etiology? An answer to the first question is that it is very unlikely that an appreciable proportion of patients have atherosclerosis because of a complete absence of apoE, since only three families have been described with such an absence (8–10). However, the phenotype in mice does not depend on complete absence of apoE. Thus, when mice heterozygous (+/−) for the *Apoe* gene disruption were fed the high fat/high cholesterol diet for 3 months, 9/9 developed lesions. Most of the lesions were fatty streaks, but fibrous cap formation was seen in the heterozygote that had the largest lesion. Only 3/9 wild-type (*Apoe* +/+) animals fed the diet had lesions, which averaged ≈1/10th the size of those in the heterozygous animals; none were complex (11). van Ree *et al.* (12) have also studied heterozygous mice (*Apoe* +/−) and have demonstrated diet-induced hypercholesterolemia and atherosclerosis in them. A hypothesis stemming from these findings is that genetic reduction in apoE levels in humans may constitute a risk factor for atherogenesis, particularly under dietary stress.

The second question, concerning the relevance of the mouse condition generated by mutation of a single gene to the human disease, which is genetically complex, can be addressed by comparing the lesions seen under various circumstances. As described above, the complex lesions in the apoE-deficient mice are very like the complex lesions seen in humans. Similarly, atherosclerotic lesions that occur spontaneously in pigs having one particular polymorphic form of the apolipoprotein constituent of LDL (apoB) (13) are also very similar in histology to human lesions. Likewise, the lesions that occur in Watanabe heritable hyperlipidemic rabbits as a consequence of a defect in the gene coding for the LDL receptor (14) are comparable to the human lesions. Finally, in mice homozygous for a disrupted LDL receptor gene (15) the lesions are again of the same type, although a high fat diet is required for their development (16). The apoE-deficient mice consequently provide a source of animals that, on a normal diet, rapidly and regularly develop atherosclerotic lesions of an advanced and apparently general type that is common to many species (including humans) independent of the specific genetic etiology. This highly reproducible phenotype is now being used to investigate the effects of other genetic modifications, drug treatments, dietary factors, and the like.

Some differences in the detailed effects of a targeted mutation in mice and of a comparable mutation in humans are nevertheless to be expected and may be very informative. For example, vascular occlusions in human patients with advanced atherosclerosis are frequently the consequence of thrombosis initiated by plaque fissuring, which exposes extracellular debris to the clotting system. No evidence of thrombotic incidents or sequelae has been reported in the ApoE-deficient mice. This suggests the presence in the mouse of protective factors or the absence of deleterious factors present in humans. Identification of such factors would be of considerable interest.

Effects of Other Gene Modifications on Atherogenesis. Thanks to the pioneering work of Brown and Goldstein on familial hypercholesterolemia (17), defects in the gene coding for the LDL receptor are the best documented genetic causes of premature atherosclerosis and coronary heart disease in humans. It was therefore to some degree unexpected that mice lacking the LDL receptor, generated by Ishibashi *et al.* (15), had only mild pathological manifestations when fed normal mouse chow, with only about twice normal levels of plasma cholesterol. Severe lesions were, however, seen when the mice were fed an atherogenic diet (16). This result is in contrast to humans with familial hypercholesterolemia due to lack of the LDL receptor; homozygous humans with this condition can have >5 times normal plasma cholesterol and develop severe atherosclerosis even in childhood.

Although they are contributing substantially to our understanding of factors that modulate lipid metabolism, none of the other single gene defects so far tested have had such a dramatic effect on atherogenesis as the apoE deficiency or absence of the LDL receptor. For example, as we had expected, gene-targeted mice lacking apoA-I have reduced HDL cholesterol levels (about one-fourth normal). In humans, lower levels of HDL cholesterol are associated with a higher risk of atherosclerosis. We were therefore somewhat surprised to find that mice having reduced levels of HDL cholesterol caused by apoA-I deficiency do not develop atherosclerotic lesions on normal chow and are no more susceptible than wild-type animals to diet-induced atherosclerosis (18). However, this finding agrees with reports that some human individuals with mutations in the *APOA1* gene that cause severe reductions in HDL cholesterol do not appear to have an increased risk of coronary artery disease (19). Thus, reduction of HDL cholesterol is most likely not sufficient to cause atherosclerosis by itself.

Combining Mutations. The success of *Apoe* gene disruption in generating severe atherosclerosis in the mouse is allowing new ways of studying the genetic complexity of atherogenesis. Thus, we and others have begun to determine how mutations in second or third genes modify the incidence and progression of the disease gen-

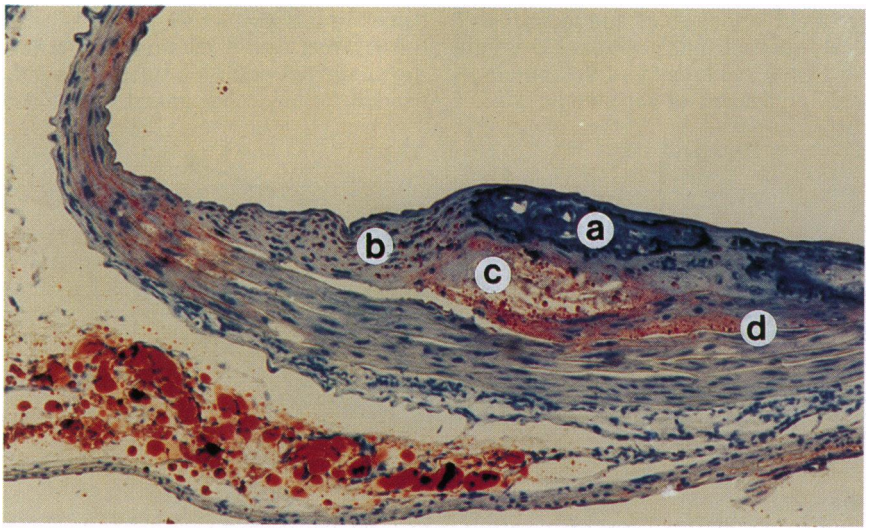

FIG. 1. A complex atherosclerotic lesion in the aorta of a 60-week-old *Apoe* −/− mouse fed regular mouse chow. a, Calcified region; b, fibrous cap; c, acellular lipid core with cholesterol crystal clefts; d, smooth muscle cells infiltrated with lipid. (×60.)

erated by the *Apoe* gene mutation. Published accounts of this type of experiment now include the protective effects on the disease of genetically increasing the levels of apoA-I (20, 21). The results show that increased levels of apoA-I synthesis mediated by a human *APOA1* transgene cause a 2- to 3-fold increase in HDL and a highly significant reduction in the incidence and size of the atherosclerotic lesions. In a similar fashion, ApoE-deficient mice are allowing evaluation of the roles of various other genetic factors involved in the detailed pathology of atherosclerosis at the level of the arterial wall, such as factors regulating smooth muscle proliferation and inflammation.

The combination in mice of two genetic manipulations, one producing a serious and complex disease and the other attempting to ameliorate its severity, is likely to be a powerful tool for investigating potential modes of gene therapy. When a human disease is the consequence of a known defect in a specific gene, the first choice for gene therapy is likely to be replacement of the defective gene function. When the disease is caused by defects in more than one gene, as is probably the case in human patients with atherosclerosis or essential hypertension, gene therapy by replacement is less practical. The possibility that effective therapy can be achieved by increasing the product of a gene (such as *Apoa1*) that is not itself the cause of the disease is therefore exciting. The apoE-deficient mouse provides a good model on which to test this type of "generic" therapy. Conceivably, therapies aimed at simultaneously increasing or decreasing production of several proteins might also be used in the future for the treatment of complex multifactorial disease.

Essential Hypertension

Essential hypertension (elevated blood pressure not associated with a recognizable predisposing condition) like atherosclerosis has a complex etiology, with genetic factors being responsible for about two-thirds of the familial aggregation of blood pressures and with transmission of cultural factors being responsible for the remaining one-third (22). Normal blood pressures are maintained by several interacting systems that control cardiac output, peripheral resistance, blood volume, renal function, and sodium balance. The renin/angiotensin system is one of these systems, and it is intimately concerned with the control of systemic and intrarenal blood pressures and sodium retention. Its relevance to essential hypertension is exemplified by the use of inhibitors of elements of the system to treat hypertension. Captopril and other orally active inhibitors of the angiotensin-converting enzyme (ACE), which converts the inactive peptide angiotensin I to the hypertensive peptide angiotensin II, are among the earliest and most successful. Losartan (DuP753), an orally active inhibitor of type 1 receptors for angiotensin II, is one of the most recent to undergo clinical trial. Virtually all the genes coding for elements in the renin/angiotensin system are consequently *a priori* candidates for involvement in hypertension.

Recent advances in the mapping of genes that affect complex phenotypes provide a second reason for selecting the renin/angiotensin system as a source of candidate genes. For example, analyses of crosses between spontaneously hypertensive stroke-prone rats and wild-type Wistar–Kyoto rats have shown that a genetic determinant of the blood pressure of animals fed a high-salt diet segregates with a chromosomal region known to include the gene coding for ACE (23, 24). Similarly, in humans, a specific variant (M235T) of the gene coding for angiotensinogen (AGT), which is the protein precursor of angiotensin I, has been found to segregate with hypertension in a sibling pair study (25). The variant was also associated with modestly elevated (120% normal) plasma AGT levels.

Linkage experiments must, however, be interpreted with caution. Although they demonstrate a genetic correlation (linkage) between the phenotype and the scored genetic variant(s), they do not in themselves prove a causative relationship between the two. In particular, they do not exclude the possibility that the significant factor is a difference in some other gene linked to the scored variant, which itself may be a neutral marker.

Gene Titration. Some of the problems associated with proving causation can be illustrated by describing gene targeting experiments initially aimed at determining whether the *AGT* variant M235T can cause hypertension in the mouse. Inspection of the published amino acid sequences of human AGT and mouse AGT quickly showed that position 235 is in a poorly conserved region of the protein. A simple equivalent of the human M235T substitution cannot therefore be made in the mouse. In its place, we decided to test the broader hypothesis advanced by Jeunemaitre *et al.* (25)—namely, that the increased level of plasma AGT associated with the variant allele was the true cause of the hypertension.

To test whether variants of the mouse *Agt* gene that directly affect plasma AGT levels also affect blood pressure, a two-part targeting strategy was used to generate animals with plasma AGT levels ranging from 0 to ≈145% normal levels. The strategy includes conventional gene targeting to produce a disrupted *Agt* gene (26) and a special form of gap-repair gene targeting to duplicate a wild-type *Agt* gene at its normal chromosomal location together with known or suspected controlling elements (27). Suitable breeding of chimeras and F_1 progeny derived from the targeted embryonic stem (ES) cells allowed generation of animals having 0, 1, 2, 3, or 4 copies of the wild-type gene. These animals had a highly satisfactory gradation of the steady-state plasma AGT levels, which spanned the relative levels seen in the human patients (Fig. 2). This method of gene titration is applicable in principle to any gene and should provide a valuable tool for analyzing quantitative genetic traits by experiments in mice.

Blood pressure measurements were made on F_2 mice having the genetically controlled gradations in plasma AGT levels. A highly significant increase in blood pressures with increasing numbers of functional *Agt* genes was observed, as illustrated in Fig. 3.

When an experiment involves more than one strain of mice, data from F_2 animals must be interpreted with caution because of possible effects from strain differences (see below). In our *Agt* experiments, these effects were avoided by comparing F_2 one-copy and three-copy animals, as is also discussed in more detail below. In this way, we were able to establish a direct causal relationship between *Agt* genotypes and blood pressures independent of any other linked or unlinked

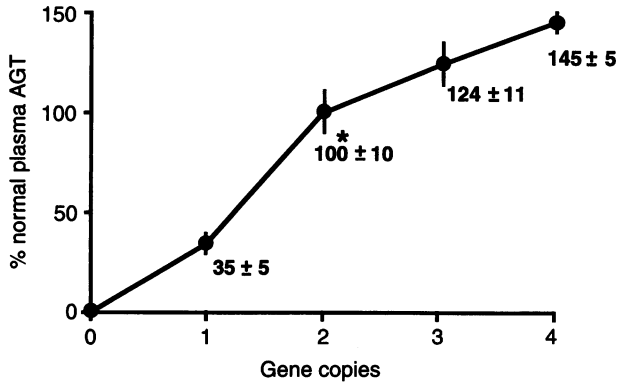

FIG. 2. Effect of *Agt* gene copy number on plasma AGT concentration relative to a normal value of 100%. Data for 1, 2, and 3 gene copies are from F_1 animals; data for 0 and 4 copies are from F_2 animals, the two-copy (wild type) mean value is assumed to be 100%.

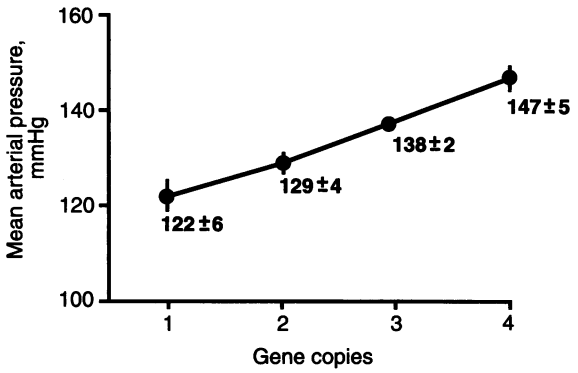

FIG. 3. Intraarterial blood pressures measured on 22 conscious and unrestrained F_2 animals having 1–4 functional copies of the *Agt* gene.

genetic differences and in animals with all their normal compensatory mechanisms intact.

Our results with the *Agt* system differ somewhat from those of Tanimoto *et al.* (28). They also disrupted the *Agt* gene but did not observe blood pressure differences between their heterozygous and wild-type mice. The investigators used ES cells derived from a B6/CBA F_1 embryo, and they mated their chimeras to outbred ICR animals. The genetic heterogeneity introduced into the experiment by these choices might have made the detection of heterozygous effects more difficult (see below).

Compensatory Changes Can Point to Other Candidate Genes. Inspection of Fig. 2 shows that single-copy F_1 animals heterozygous for a wild-type and disrupted *Agt* gene have a steady-state plasma AGT concentration (35% of normal), which is less than the 50% level that might be expected from their genotype. A simple hypothesis which would explain this finding is that pressure-sensing elements in the kidney, in attempting to restore the blood pressures of these heterozygotes to normal, increase the output of renin from the kidney, thereby increasing the proportion of plasma AGT converted to angiotensin I (renin generates angiotensin I from AGT) and thence through the action of ACE to angiotensin II. This hypothesis receives support from the finding that plasma renin activity in these animals is ≈2.5 times normal. Thus, one may reasonably anticipate that genetic changes in renin expression will also prove to influence resting blood pressures. As described below, preliminary evidence from other targeting experiments points in the same direction.

Other Genes in the Renin/Angiotensin System. We have tested two other genes in the renin/angiotensin system by gene targeting for their possible roles in the genetic control of blood pressures. One is the *Ace* gene, which encodes two forms of ACE—a somatic form present in the plasma and throughout the body and a testis form, which is specific to the testis and therefore to males. The *Ace* gene was disrupted in such a way that neither form of the enzyme could be synthesized from the targeted gene (29). Data from these experiments showed a highly intriguing result. Heterozygosity for disruption of the *Ace* gene (+/−) decreases the level of serum ACE to approximately the same extent in male and female F_1 mice (to 75% and 66% of normal, respectively). However, the blood pressures of the F_1 males are decreased (by 16–21 mmHg), but the blood pressures of the F_1 females are not decreased. (The use of F_1 animals ensures that the observed differences are solely due to the targeted mutation.) In addition, the breeding data showed that homozygosity for disruption of the *Ace* gene affects the fertility of males but not of females. These observations suggest the need for further investigation of the effects of gender and the *ACE* gene on reproduction and blood pressure regulation.

Two main types of receptors for angiotensin II (the active peptide of the renin/angiotensin system) have been described: type 1 receptors, defined by their affinity for DuP753 (Losartan); and type 2, defined by their affinity for PD 123177. The type 1 receptors have been subdivided into two subtypes, 1A and 1B, which differ in their sequences and are encoded by separate genes. Mice homozygous for a disruption of the gene (*Agtr1a*) coding for the type 1A receptor have virtually no pressor response to an intravenous injection of angiotensin II (30). Both male and female F_2 heterozygotes (+/−) show a decrease in blood pressures of ≈12 mmHg relative to wild-type F_2 (+/+) animals. Suitable control experiments showed that this lowering of blood pressure in the heterozygous mutants is independent of any genetic differences between the strains of mice used. Thus, all three genes in the renin/angiotensin systems that have been modified by gene targeting can directly cause changes in resting blood pressure.

Unexpected phenotypes were seen in two of the homozygous mutants. The *Agt* null and *Ace* null animals share an interesting kidney pathology characterized by a generalized thinning of the cortex, with focal areas of severe atrophy (26, 29). An unexpected thickening, in some cases to near occlusion, is observed in the walls of many small arteries in the kidney, but not elsewhere, and may be the primary defect. Interestingly, animals lacking the type 1A angiotensin II receptor survive well and do not show these abnormalities (30).

Genes in Other Blood Pressure Regulating Systems. Many of the actions of angiotensin II are antagonized by the atrial natriuretic peptide (ANP), a peptide produced primarily in the cardiac atria. Genetic factors leading to a decrease in ANP function are consequently also candidate causes of hypertension. We therefore generated mice with a targeted disruption of the gene (*proANP*) coding for the precursor of ANP, again with ES cells derived from strain 129 and with subsequent breeding of chimeras to strain B6 mates (31). Mutant F_2 heterozygotes (+/−) had atrial ANP levels close to half those of wild-type F_2 (+/+) animals. However, neither the circulating ANP levels nor the intraarterial blood pressures of the (+/−) and (+/+) animals differed significantly when they were fed regular mouse chow (0.5% NaCl). On the other hand, when the animals were fed a high-salt diet (8% NaCl) for 2 weeks, the blood pressures of the F_1 heterozygotes increased significantly ($P < 0.01$) from 118 mmHg on normal chow to 146 mmHg on the high-salt diet, while the blood pressures of the wild-type F_1 mice were unchanged by the diet. Again, the use of F_1 animals ensures that the observed differences are solely due to the targeted mutation. These experiments consequently establish that genetically reduced ANP can cause salt-sensitive hypertension.

An interesting, although unexpected, effect of disrupting the endothelin 1 gene by targeting has been reported by Kurihara *et al.* (32). Endothelin 1 is a 21-amino acid vasoconstrictive peptide. Homozygous mutants (−/−) died at birth consequent to craniofacial defects. Conscious and unrestrained heterozygotes (+/−) had mean intraarterial blood pressures (116 mmHg) ≈11 mmHg above the pressures (105 mmHg) of their wild-type littermates. The observation that a genetic reduction of endothelin 1 levels (to ≈60% normal in the lungs of the heterozygotes) caused an increase in blood pressure was unexpected because of the known pressor effects of the endothelin 1 peptide.

Experimental Considerations

The Choice of Proper Controls. In carrying out gene targeting, most investigators including ourselves start with ES cells derived from inbred strain 129. The ideal way to ensure that the observed phenotypes are due solely to the targeted gene and not to polymorphic strain differences

between the animals under study is to breed the chimeras to mates of the same inbred strain 129. We therefore regularly do this to ensure the future availability of the targeted mutation in the inbred strain 129 background. Unfortunately, confining all subsequent breeding to strain 129 is impractical if many animals are required because of the poor breeding characteristics of the strain and its susceptibility to infection. These problems point to the need for easily targetable ES cells from inbred strains that are more fecund and hardy than strain 129.

In practice, as described above, we normally mate the chimeras generated from inbred strain 129-targeted ES cells to another inbred strain, typically B6, to obtain first generation progeny (F_1). (Heterozygotes obtained from breeding the chimeras to strain 129 mates can be used for this purpose when chimeras cease to be available.) These hybrid F_1 animals are vigorous and fecund, and the animals of each sex are genetically identical except for the gene (wild type or targeted) they receive from the strain 129-derived ES cell. Comparisons between wild-type (+ chromosome from B6/+ chromosome from 129) and heterozygous (+ chromosome from B6/targeted chromosome from 129) F_1 animals therefore provide a test of the effects of targeted modifications on the phenotype of interest completely free from any genetic heterogeneity introduced by the use of two strains (Fig. 4).

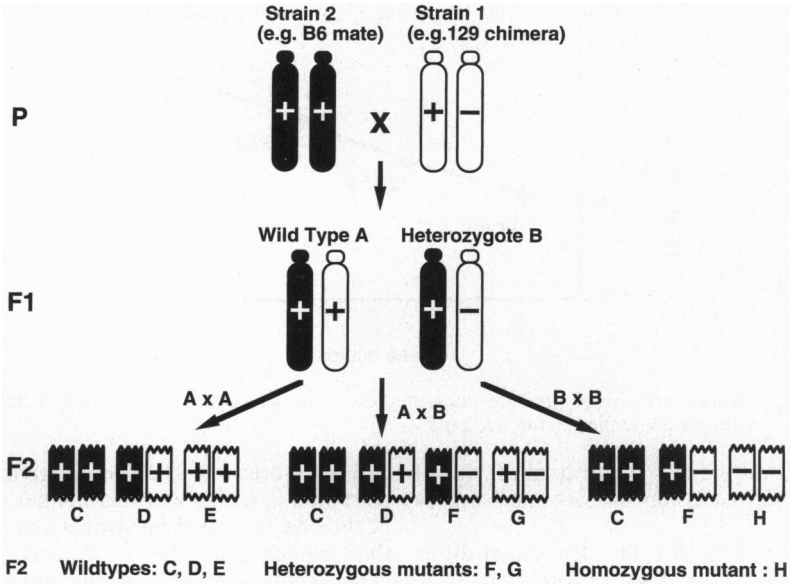

FIG. 4. Cosegregation of strain-specific chromosomal regions and the target locus influences experimental design and the choice of controls when two inbred strains are used in the breeding. Comparison of F_1 animals, wild-type A and mutant heterozygote B, allows assessment of the effects of the targeted mutation free from biases due to any genetic differences between the two strains. Note that there are three classes of F_2 wild-type animals (C, D, and E) and two classes of F_2 heterozygotes (F and G), which can all be distinguished in ways that are discussed in the text. Comparison of the three classes of F_2 wild-type animals allows assessment of the effects on the trait of interest of any naturally occurring strain differences at the target locus or in any genes linked to it. Wild-type animal D is an excellent control for the mutant heterozygote F, as is wild-type animal E for the mutant heterozygote G and for the mutant homozygote H. Effects of strain differences in linked genes could be avoided in future generations if only class E, G, and H animals are mated. P, parental animals: usually a chimera derived from strain 1-targeted ES cells (typically strain 129) and a strain 2 mate (typically B6). F_1, first generation offspring. F_2, three sets of second generation offspring that can be derived from intercrossing F_1 animals. Strain 1-derived chromosomes carrying the targeted (−) or the wild-type (+) gene at the target locus are shown in white. Homologous chromosomes from the second strain are shown in black; all carry a wild-type gene (+) at the target locus. F_2 progeny chromosomes are shown without centromeres and telomeres to signify that crossovers reduce the extent of cosegregation to linked regions rather than whole chromosomes.

Effect of Genes Unlinked to the Targeted Locus. Proceeding to the F_2 generation by intercrossing F_1 +/− animals makes it necessary to consider the effects on the phenotype of differences between the two parental strains at loci other than the target locus. Any genes that differ between the two strains and are not linked to the target locus will in general segregate randomly within F_2 animals. If any of these unlinked differences affect blood pressure, for example, they will increase the spread of blood pressures observed in different animals, even though they have identical genotypes at the target locus. Differences in unlinked genes consequently make it more difficult to detect subtle effects of the targeted gene, although they do not affect the average magnitude of the effect.

Experiments with the *proANP* locus illustrate how this spread of the phenotype in F_2 animals can be used in an advantageous way to identify other genes likely to be important. Some strains of mice (including B6) naturally have one copy per haploid genome of a gene that codes for renin; other strains (including strain 129) naturally have two renin genes per haploid genome (33, 34). The renin locus *Ren* is not linked to the *proANP* locus. Typing animals in the *proANP* targeting experiment for *Ren* genotypes showed that those F_2 animals that by chance received both their *Ren* loci from strain 129 (and therefore have four copies of a renin gene) have higher blood pressures when fed 2% salt than animals of the same *proANP* genotype that received three copies. These in turn have pressures higher than the two-copy animals that received both *Ren* loci from B6 (John H. Krege and Simon W. M. John, personal communication). These preliminary experiments do not eliminate the possibility that in this case the spreads in phenotype are not in part or completely due to strain differences in genes linked to the *Ren* locus, but they do show how the effects of segregation of a second chromosomal region unlinked to the target locus can be detected and how it can influence the phenotype resulting from the planned change at the target locus.

Effect of Genes Linked to the Target Locus. Polymorphic differences in genes linked to the target locus can cause more serious complications. The frequency with which related genes are linked to target loci is much greater than would be expected by chance because related genes often occur as linked clusters in the mammalian genome. For example, three mouse apolipoprotein genes—*Apoa1*, *Apoc3*, and *Apoa4*—are all within a 20-kb stretch of DNA (unpublished data). Similarly, two mouse natriuretic peptide precursor genes—*proANP* and *proBNP*—are within 20 kb (Mark E. Steinhelper, personal communication).

Polymorphism in a related linked gene is unimportant if the polymorphism has no effect on the phenotype of interest. But this will no longer be true if the linked polymorphic allele affects the phenotype. Imagine, for example, that strain 129 has a linked polymorphic allele that affects the phenotype under study positively when compared to the corresponding allele in strain B6. The effects of any negative mutations at the target locus will in this case be underestimated (or even reversed) in a conventional comparison of F_2 progeny derived from intercrossing F_1 heterozygotes. This is because all the F_2 progeny that are homozygous mutant (−/−) at the target locus will also be homozygous for the strain 129-derived (more positive) allele at the linked locus (Fig. 4). Likewise all the F_2 progeny from this intercross that are wild type (+/+) at the target locus will also be homozygous for the B6-derived (less positive) allele at

the linked locus. Thus, polymorphisms at linked phenotype-related loci can alter the apparent magnitude of the effects of the targeting if only conventional F_2 +/+, +/−, and −/− progeny derived from intercrossing F_1 +/− animals are compared. Conceivably, they could even reverse its sign, or give an apparent effect from the targeting when none is there. Linked genes will not, however, change the spread of values within a specific target locus genotype.

In gene knockout experiments in which the effects of absence of the target gene product are extreme and overriding, complications from linked strain differences are usually of little concern (as in the case of *Apoe* −/− mice). However, when the phenotype is a quantitative variable and the effects may be subtle, rigorous controls in which the targeted mutation is the sole variable become important. Fig. 4 illustrates some of the complexities introduced by the use of two strains, even when both are inbred. For example, the figure illustrates that there are three classes (C, D, and E) rather than one class of F_2 wild-type animals. All are +/+ at the target locus, but they differ in the sources of their wild-type alleles. Likewise, there are two classes (F and G) of mutant F_2 heterozygote (+/−). Choice of rigorous controls requires careful consideration of these classes, as indicated in the legend to Fig. 4. The different classes of F_2 animals can readily be distinguished if a natural polymorphism can be found between the two strains at the target locus. Otherwise, it may be necessary to use simple sequence length polymorphisms linked to the target locus in order to identify them—a strategy that was used in a related situation to distinguish three-copy and four-copy F_2 animals in our *Agt* gene experiments (26).

There are other ways of avoiding the effects of strain differences in genes linked to the target locus. For example, in the gene titration experiment carried out at the *Agt* locus, we compared the one-copy and three-copy animals (26). The one-copy animals and the three-copy animals both have a wild-type *Agt* allele and nearby DNA derived from strain B6, and both have a targeted *Agt* allele and nearby DNA derived from strain 129; they consequently differ systematically only at the targeted locus.

This technical discussion is intended to emphasize that, even in a complex genetic system, gene targeting experiments can with careful attention to details be designed to prove that a specific phenotype is caused by the targeted change and not by any other differences between the participating strains. This is the elusive proof of causation referred to previously. Once this proof has been secured, it may not be necessary to continue to preserve the genetic "purity" of a mutant strain. Alternatively, by backcrossing to an inbred strain (such as B6) the genetic purity of a targeted mutant can be recovered. The resulting mutant strain will be inbred B6 except for being homozygous for a small region derived from the ES cell strain that includes and surrounds the target locus. The complications associated with polymorphisms in genes closely linked to the target loci therefore still remain, unless backcrossing is also carried out to secure animals having the ES cell-derived wild-type gene at the target locus, but the complications associated with polymorphisms elsewhere in the genome are eliminated. Consequently, when the effects of combining targeted mutations at several loci are to be investigated, animals backcrossed to B6 (or any other chosen inbred strain) are very valuable.

Conclusions

How are gene targeting experiments in mice helping to develop a better understanding of the genetic etiologies of atherosclerosis and essential hypertension, and what can be expected from them in the future?

At present, the most significant contribution toward this understanding for atherosclerosis is probably the generation of precisely defined mouse models of the disease: mice with disruption in the genes coding for apoE or the LDL receptor. These models are allowing assessment of the effects of other genes on atherogenesis by using the powerful genetic tools available in the mouse. Carefully controlled investigations of the effects of diet or of drugs are also facilitated by these animals.

At present, the most significant contribution to understanding the genetic etiology of essential hypertension is probably the demonstration that discrete alterations in the expression of a variety of different genes can individually cause changes in the blood pressures of mice, even when the mice have all their compensatory mechanisms intact. These effects are readily detected in animals having moderate decreases in gene function due to heterozygosity for gene disruptions or modest increases due to gene duplications.

Considerable work still remains to be done in dissecting out in a rigorous manner the effects of alterations in single genes on the induction or progression of atherosclerosis and on the control of blood pressures. Perhaps even more exciting is the opportunity now becoming available to breed animals in which the effects of precise differences in more than one gene can be studied in combination. It would be overly optimistic to expect that all data from the mouse will directly apply to humans. Some species differences are to be expected, but it will be important not to dismiss unexpected findings too easily. Indeed, determining the basis of the unexpected findings with the help of mouse genetics may provide important insights into the causes and treatment of these complex multifactorial diseases.

We thank Tom Coffman, Beverly Koller, Kathy Sulik, and the members of our laboratory for their help in preparing this review. Our work is supported by grants from the National Institutes of Health (GM20069, HL42630, and HL49277).

1. Ross, R. (1993) *Nature (London)* **362,** 801–809.
2. Gordon, T., Castelli, W. P., Hjortland, M. C., Kannel, W. B. & Dawber, T. R. (1977) *Am. J. Med.* **62,** 707–714.
3. Paigen, B., Morrow, A., Brandon, C., Mitchell, D. & Holmes, P. (1985) *Atherosclerosis* **57,** 65–73.
4. Zhang, S. H., Reddick, R. L., Piedrahita, J. A. & Maeda, N. (1992) *Science* **258,** 468–471.
5. Plump, A. S., Smith, J. D., Hayek, T., Aalto-Setälä, K., Walsh, A., Verstuyft, J. G., Rubin, E. M. & Breslow, J. L. (1992) *Cell* **71,** 343–353.
6. Reddick, R. L., Zhang, S. H. & Maeda, N. (1994) *Arterioscler. Thromb.* **14,** 141–147.
7. Nakashima, Y., Plump, A. S., Raines, E. W., Breslow, J. L. & Ross, R. (1994) *Arterioscler. Thromb.* **14,** 133–140.
8. Ghiselli, G. E., Schaefer, E. J., Gascon, P. & Brewer, H. B. (1981) *Science* **214,** 1239–1241.
9. Mabuchi, H., Itoh, H., Takeda, M., Kajinami, K., Wakasugi, T., Koizumi, J., Takeda, R. & Asagami, C. (1989) *Metabolism* **38,** 115–119.
10. Kurosaka, D., Teramoto, T., Matsushima, T., Yokoyama, T., Yamada, A., Aikawa, T., Miyamoto, Y. & Kurokawa, K. (1991) *Atherosclerosis* **88,** 15–20.
11. Zhang, S. H., Reddick, R. L., Burkey, B. & Maeda, N. (1994) *J. Clin. Invest.* **94,** 937–945.
12. van Ree, J. H., van den Broek, W. J. A. A., Dahlmans, V. E. H., Groot, P. H. E., Vidgeon-Hart, M., Frants, R. R., Wieringa, B., Havekes, L. M. & Hofker, M. H. (1994) *Atherosclerosis* **111,** 25–37.
13. Rapacz, J., Hasler-Rapacz, J., Taylor, K. M., Checovich, W. J. & Attie, A. D. (1986) *Science* **234,** 1573–1577.
14. Buja, L. M., Kita, T., Goldstein, J. L., Watanabe, Y. & Brown, M. S. (1983) *Arteriosclerosis* **3,** 87–101.
15. Ishibashi, S., Brown, M. S., Goldstein, J. L., Gerard, R. D., Hammer, R. E. & Herz, J. (1993) *J. Clin. Invest.* **92,** 883–893.
16. Ishibashi, S., Goldstein, J. L., Brown, M. S., Herz, J. & Burns, D. K. 91994) *J. Clin. Invest.* **93,** 1885–1893.
17. Brown, M. S. & Goldstein, J. L. (1986) *Science* **232,** 34–47.
18. Li, H., Reddick, R. L. & Maeda, N. (1993) *Arterioscler. Thromb.* **13,** 1814–1821.
19. Assmann, G., von Eckardstein, A. & Funke, H. (1993) *Circulation* **87,** Suppl. III, 28–34.
20. Pászty, C., Maeda, N., Verstuyft, J. & Rubin, E. M. (1994) *J. Clin. Invest.* **94,** 899–903.
21. Plump, A. S., Scott, C. J. & Breslow, J. L. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9607–9611.
22. Ward, R. (1990) in *Hypertension: Pathophysiology, Diagnosis, and Management*, ed. Laragh, J. H. & Brenner, B. M. (Raven, New York), pp. 81–100.

23. Hilbert, P., Lindpaintner, K., Beckmann, J. S., Serikawa, T., Soubrier, F., Dubay, C., Cartwright, P., De Gouyon, B., Julier, C., Takahasi, S., Vincent, M., Ganten, D., Georges, M. & Lathrop, G. M. (1991) *Nature (London)* **353,** 521–529.
24. Jacob, H. J., Lindpaintner, K., Lincoln, S. E., Kusumi, K., Bunker, R. K., Mao, Y.-P., Ganten, D., Dzau, V. J. & Lander, E. S. (1991) *Cell* **67,** 213–224.
25. Jeunemaitre, X., Soubrier, F., Kotelevtsev, Y. V., Lifton, R. P., Williams, C. S., Charru, A., Hunt, S. C., Hopkins, P. N., Williams, R. R., Lalouel, J.-M. & Corvol, P. (1992) *Cell* **71,** 169–180.
26. Kim, H.-S., Krege, J. H., Kluckman, K. D., Hagaman, J. H., Hodgin, J. B., Best, C. F., Jennette, J. C., Coffman, T. M., Maeda, N. & Smithies, O. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 2735–2739.
27. Smithies, O. & Kim, H.-S. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 3612–3615.
28. Tanimoto, K., Sugiyama, F., Goto, Y., Ishida, J., Takimoto, E., Yagami, K., Fukamizu, A. & Murakami, K. (1994) *J. Biol. Chem.* **269,** 31334–31337.
29. Krege, J. H., John, S. W. M., Langenbach, L. L., Hodgin, J. B., Hagaman, J. R., Bachman, E. S., Jennette, J. C., O'Brien, D. A. & Smithies, O. (1995) *Nature (London)*, in press.
30. Ito, M., Oliverio, M. I., Mannon, P. J., Best, C. F., Maeda, N., Smithies, O. & Coffman, T. M. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3521–3525.
31. John, S. W. M., Krege, J. H., Oliver, P., Hagaman, J. R., Hodgin, J. B., Pang, S. C., Flynn, T. G. & Smithies, O. (1995) *Science* **267,** 679–681.
32. Kurihara, Y., Kurihara, H., Suzuki, H., Kodama, T., Maemura, K., Nagai, R., Oda, H., Kuwaki, T., Cao, W.-H., Kamada, N., Jishage, K., Ouchi, Y., Azuma, S., Toyoda, Y., Ishikawa, T., Kumada, M. & Yazaki, Y. (1994) *Nature (London)* **368,** 703–710.
33. Piccini, N., Knopf, J. L. & Gross, K. W. (1982) *Cell* **30,** 205–213.
34. Panthier, J.-J., Holm, I. & Rougeon, F. (1982) *EMBO J.* **1,** 1417–1421.